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Identification and localization of calcium-dependent protease II in *Neurospora crassa* and *Uromyces appendiculatus*

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Summary. The existence of Ca^{2+}-dependent protease II in crude extracts of *Neurospora crassa* and *Uromyces appendiculatus* was demonstrated by immunoblotting using specific antibodies. In both extracts two immunoreacting bands were observed. The molecular mass of the major band in *N. crassa* corresponded to 37 kDa, while that in *U. appendiculatus* was 43 kDa, similar to that previously reported for *Allomyces arbuscula*. Immunofluorescence of the enzyme was predominantly localized in the apical regions of germlings and growing hyphae, suggesting a functional role for the enzyme in hyphal growth.

Keywords: Calcium-dependent protease; Western blot; Immunofluorescence localization; Fungal hyphae.

Introduction

Changes in Ca^{2+} concentrations are thought to regulate the activities of various enzymes. Ca^{2+}-dependent proteinases (CDPs; called calpains in mammalian cells) are cytosolic neutral cysteine proteinases and have been found in a wide variety of organisms including vertebrates (Johnson and Guttmann 1997), invertebrates (Pintér and Friedrich 1988), plants (Safadi et al. 1997), and fungi (Ojha and Wallace 1988, Denison et al. 1995, Ojha 1996a). In mammalian cells two types of calpains, requiring micro- and millimolar concentrations of Ca^{2+} for activation of in vitro catalytic activities (μ- and m-calpains) have been identified. Sorimachi et al. (1996) have identified a third type of calpains, which is tissue specific. These enzymes are considered to be involved in the regulation of many cellular functions by limited proteolysis. The physiologically important substrates for calpains are not known with certainty; however, evidence indicates three different groups of proteins as targets: cytoskeletal proteins, membrane-bound enzymes, and protein kinases.

Clearly, the diversity of identified substrates illustrates the importance of understanding the mechanism(s) involved in the precise function of calcium-dependent proteases. Among the cytoskeletal proteins various forms of intermediate filaments (vimentin, desmin), tubulins and microtubule-associated proteins (MAPs), actin and a large number of actin-binding proteins (talin, filamin, and foldrin) appear to be highly susceptible substrates of calpains (for reviews, see Billger et al. 1988, Croall and DeMartino 1991, Anderson et al. 1996, Sorimachi et al. 1996, Springer et al. 1997).

A Ca^{2+}-dependent cysteine protease (CDP II) was purified from *Allomyces arbuscula*, an aquatic fungus, and found to be localized mainly in the apical regions of the growing hyphae. Its presence and absence was shown to be related to growth (Ojha and Wallace 1988; Ojha 1990, 1996b; Huber and Ojha 1994; Huber 1996). The enzyme appeared at the time of germination and accumulated during the growth phase but disappeared when growth ceased and the apex was transformed into a reproductive structure (Ojha 1996b). Many of the functional properties of this enzyme are reminiscent of the extensively characterized mammalian

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calpains, although there are some significant differences, particularly the restricted requirement of P1 amino acid by the fungal enzyme. Therefore, it is suggested that the action and regulation of this enzyme may be similar to the mammalian calpains. We have therefore extended our studies to other fungi and report that antigens similar to Allomyces Ca^{2+}-dependent protease are present in germinating cells of *Neurospora crassa* and *Uromyces appendiculatus*, two widely studied model fungal systems.

Material and methods

Organism and culture conditions

Wild-type *Neurospora crassa* (FG 5262, strain St. Lawrence 74A) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kans. In order to produce a large number of macroconidia from *N. crassa*, the fungus was first grown on solid Vogel's minimal medium (Vogel 1956) for 3 days at 33 °C in the dark and then at 25 °C in artificial light for 4 days. Conidia were harvested and germinated at an inoculum density of 5×10^6 conidia/ml in liquid Vogel's medium containing 1.5% sucrose at 37 °C for different periods (0, 2, 6, and 12 h) on a rotary shaker (150 rpm).

Uredospores of *Uromyces appendiculatus*, race O, were harvested from infected greenhouse-grown bean leaves (*Phaseolus vulgaris* L. var. Pinto) and stored at 4 °C until used for experiments. Prior to use, they were exposed to vapors of β-ionone (4-[2,6,6,-trimethyl-1-cyclohexen-1-yl]-3-buten-2-one; Sigma Chemical Co., St Louis, Mo.) for at least 20 min to overcome the activity of natural germination self-inhibitors as described by French et al. (1977). The β-ionone-treated uredospores were dispersed onto either smooth polystyrene membranes or polystyrene membranes bearing 0.5 µm high topographies prepared as described earlier (Hoch et al. 1987, Kwon et al. 1991), misted with distilled-deionized water (ddH_2O) and left in a humidified chamber for 20 min, then air dried. This procedure enhanced adherence of the uredospores to the substrata. The spore-laden membranes were floated on ddH_2O with spore side down for 3 h at 17 °C. The germinated uredospores were then prepared for immunofluorescence as described below.

Fixation, wall digestion, and plasma membrane permeabilization

Conidia and mycelia of *N. crassa* as well as germlings of *U. appendiculatus* were fixed with 3.7% paraformaldehyde in 50 mM phosphate buffer, pH 7.4, for 30 min at room temperature. Partial digestion of the cell wall was done by incubating the cells with 5 mg of Novozym 234 (Sigma) per ml in phosphate buffer, pH 6.5, for 1–2 min at room temperature and stopped by rinsing the cells three times with phosphate buffer. The cells were then permeabilized with 0.5% Triton X-100 in the same buffer for 15 min at room temperature. Triton was removed to washing five times in phosphate buffer. For *N. crassa*, the cells were allowed to settle onto 0.1% poly-L-lysine-coated slides, but polystyrene-attached *U. appendiculatus* germlings were processed intact.

Immunofluorescence of Ca^{2+}-dependent protease

The distribution of CDP II was examined with polyclonal antibodies prepared against Ca^{2+}-dependent protease purified as described by Huber and Ojha (1994). Slides treated with 0.1% poly-L-lysine (molecular weight, 300,000; Sigma) in ddH_2O were loaded with 50–100 µl of the conidial or mycelial suspension. The cells were allowed to settle onto the slide for 30 min. They were incubated with phosphate buffer containing 1% bovine serum albumin (BSA) and 1% nonfat commercial milk powder followed by incubation with CDP II polyclonal antibody diluted to 1 : 100 in 50 mM phosphate buffer for 2–4 h at 37 °C. Following a rinse in phosphate buffer, the material was incubated for 2 h at 37 °C with fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin (Sigma) diluted 1 : 100. After a final rinse in phosphate buffer, the cells were mounted in *p*-phenylenediamine-glycerol (10%, w/v). An Orthoplan epiillumination microscope (Ernst Leitz, Wetzlar, Federal Republic of Germany) equipped with fluotar optics and selective filter combinations was used to visualize the FITC fluorescence patterns. Photographs were taken on 400 ASA HP5 black-and-white film (Ilford, Basel, Switzerland). The immunofluorescence pictures shown in Fig. 3 were taken by Hamamatsu color, chilled 3 CCD camera. The images were developed by RasterOps video capture and treated by Adobe Photoshop 3.0 programme.

Protein extraction and determination

10 g of uredospores of *U. appendiculatus* previously hydrated overnight and treated with β-ionone vapors for 15 min were germinated for 3 h in 1.5 liter of stirred ddH_2O (Nanopure II system; Dybron/Bairnsted, Boston, Mass.) containing 50 µl of β-ionone (Moloshok et al. 1993). Following germination, the germlings were filtered through a 70 µm pore size mesh Nitex cloth (Tetko, Inc., Briarcliff Manor, N.Y.) and frozen in liquid nitrogen. The germlings of *U. appendiculatus* and *N. crassa* (prepared as described earlier) were ground in liquid nitrogen in a mortar with approximately 1 g of 500 µm diameter glass beads (Sigma). The frozen powder was suspended in 1–5 ml of MOPS (morpholinepropanesulfonic acid) extraction buffer, pH 7, containing 20 ml of MOPS, 2 mM EDTA, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 3 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.25 µM pepstatin, 5 µM leupeptin, and 200 µM aprotinin, and the homogenate was centrifuged at 20,000 rpm for 30 min at 4 °C and the supernatant recovered. Protein concentration in the supernatants was determined by the method of Spector (1978).

One-dimensional gel electrophoresis and immunoblotting

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) according to Laemmli (1970). Total extracted proteins (30–50 µg/slot) were electrophoresed along with the following standard molecular-mass markers: phosphorylase b, 94000; albumin, 67000; ovalbumin 43000; carbonic anhydrase 30000; trypsine inhibitor, 20100; lactalbumin, 14400 Da (all Pharmacia, Uppsala, Sweden).

Proteins from gels were electrophoretically transferred to nitrocellulose paper BA 85 (pore size, 0.45 µm; Schleicher and Schuell GmbH, Dassel, Federal Republic of Germany) with the transfer buffer described by Burnette (1981). The transferred proteins were stained with 0.05% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid (TCA). The nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline (TBS) for 4 h at room temperature or overnight at 4 °C with polyclonal antibodies raised in rabbit against Ca^{2+}-dependent protease II (CDP II) from *A. arbuscula*. The antibodies were diluted to 1 : 2,000 in TBS containing 0.5% BSA. After 4 washes in TBS, incubation with the secondary antibody, peroxidase-labeled anti-rabbit immunoglobulin G (IgG) (Amersham, Little Chalfont, England), was performed for 1 h at room

temperature. Following 4 washes in TBS, peroxidase activity was assessed with 0.5 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Fluka Chemie, Buchs, Switzerland) per ml in 100 mM Tris-HCl, pH 7.5, containing 0.03% H_2O_2.

Results and discussion

Immunoblots of crude protein extracts from exponentially growing *A. arbuscula*, *N. crassa*, and *U. appendiculatus*, separated by one-dimensional SDS-polyacrylamide gels revealed two bands at molecular masses of 43 and 40 kDa in *A. arbuscula* (Fig. 1, lane a) and one each in *N. crassa* and *U. appendiculatus* corresponding to a molecular mass of 37 and 43 kDa, respectively (Fig. 1, lanes b and c). These results demonstrated the presence of immunoreactive proteins in *N. crassa* and *U. appendiculatus* specific to *A. arbuscula* CDP II antibodies.

The specificity of the immunoreactions was tested with antibody solutions stripped off the CDP II-specific IgGs by preincubation with varying concentrations of purified enzyme from *A. arbuscula*. As shown in Fig. 2, the immuno-adsorption of antibodies suppressed the appearance of immunoreacting bands in a concentration-dependent manner in all the three organisms. Additional controls were done with pre-immune serum and in these cases too, immunoreacting bands were absent (results not shown). These results taken together indicated that the bands revealed with CDP II antibodies in *N. crassa* and *U. appendiculatus* were indeed specific and shared epitopes with *A. arbuscula* antigen.

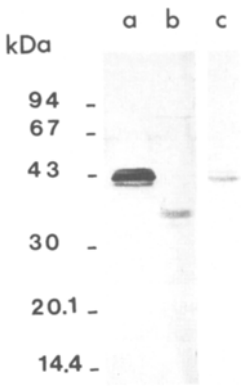

Fig. 1. Western blot of crude protein extracts (30 µg/slot) from germlings of *A. arbuscula*, *N. crassa*, and *U. appendiculatus*. Proteins were immunoblotted with an affinity-purified antibody prepared from *A. arbuscula* CDP II. *a A. arbuscula* protein extract showing 43 and 40 kDa bands; *b N. crassa*, 37 kDa band; *c U. appendiculatus*, 43 kDa band

The SDS-PAGE of crude extract from *A. arbuscula* revealed three immunoreactive bands corresponding to relative molecular mass of 45, 43, and 40 kDa (Ojha unpubl. results); however, the purified preparation invariably showed two bands at molecular masses of 43 and 40 kDa, respectively (Ojha 1990, Ojha and Favre 1991). The 45 kDa band in the SDS-PAGE of crude extracts was always present regardless of whether or not the immunoblots were made with 43 or 40 kDa peptide-specific antibodies. Both antibodies show strong cross-reactivity in immunoblots (Ojha et al. 1999). However, the 45 kDa protein does not co-purify with either the 43 or 40 kDa enzyme (unpubl. results).

Although the relative molecular mass of the Uromyces antigenic protein agreed closely with the Allomyces enzyme, that of *N. crassa* was considerably smaller (37 kDa). This is not unique to Allomyces Ca^{2+}-dependent protease. Differences in the relative molecular weight of the extensively studied animal Ca^{2+}-dependent proteases (calpains) have been observed in preparations from different origins. A great majority of the well characterized CDPs from animals have been reported to exist as heterodimers with 80 kDa (catalytic) and 30 kDa (regulatory) subunits; however, wide variations in the molecular weight of many CDPs from different origins have also been reported. For example, Zimmerman and Schlaepfer

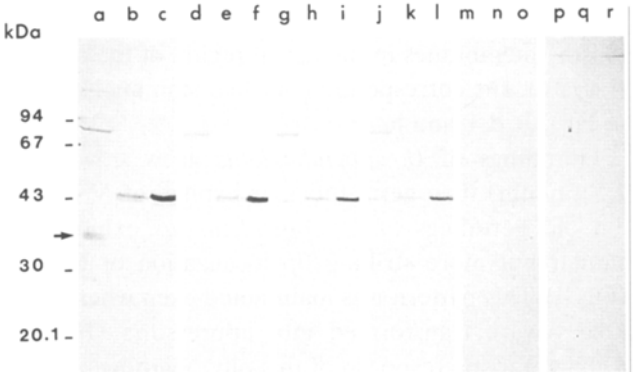

Fig. 2. Specificity of *A. arbuscula* CDP II polyclonal antibodies in *N. crassa*, *U. appendiculatus*, and *A. arbuscula* Western blot reactions. The antibodies were pre-incubated with varying concentrations of purified homologous CDP II enzyme for 30 min at room temperature prior to their use in immunoblot reactions. *a*, *d*, *g*, *j*, *m*, and *p* immunoblots of *N. crassa* (CDP II band shown with arrow); *b*, *e*, *h*, *k*, *n*, and *q* *U. appendiculatus*; *c*, *f*, *i*, *l*, *o*, and *r* *A. arbuscula*. Antibodies-to-enzyme ratio in preincubation: *a–c* control (antibody without preincubation with enzyme); *d–f* 1 : 0.3; *g–i* 1 : 0.55; *j–l* 1 : 0.83; *m–o* 1 : 1.11; *p* and *r* 1 : 5.55 (note the progressive decrease of the intensity and finally the disappearance of the bands)

(1984) purified three CDPs from skeletal muscle and brain tissues with molecular masses of 154, 96, and 76 kDa and Mykles and Skinner (1986) purified four CDPs from lobster claw and abdominal muscle and determined their molecular masses to be 310, 195, 125, and 59 kDa. The molecular masses of two cytosolic CDPs from rat liver were found to be 110 and 80 kDa (Pontremoli et al. 1984). Monomeric calcium-dependent neutral protease with a molecular mass of 80 kDa has also been reported in erythrocytes (Molinari et al. 1994). Therefore, the difference in the relative molecular mass observed between distantly related *A. arbuscula*, (43 and 40 kDa), a phycomycete, *N. crassa* (37 kDa), an ascomycete, and *U. appendiculatus* (43 kDa), a basidiomycete, is not surprising considering the evolutionary distance that separates these species.

Distribution of Ca^{2+}-dependent protease

The immunofluorescence localization of CDP II showed that its distribution was isodiametric in non-germinated conidia (Fig. 3a) but it became highly polarized during germination (Fig. 3c, e). The localization was exclusively in the apical regions where it appeared as a cap on the tip of the germ tubes. This pattern of localization was maintained for up to 6 h of germination (Fig. 3e). However, in hyphae from 12 h old cultures, which corresponds to late exponential-growth phase, the localization pattern changed and the enzyme appeared as dispersed punctate dots along the plasma membranes in the apical region of the hyphae (Fig. 3g). The corresponding nuclear staining is shown in Fig. 3b, d, f, and h.

Germlings of *U. appendiculatus* grow slower (ca 1.5 μm/min) than germ tubes or hyphae of *N. crassa*. 3 h old germlings of *U. appendiculatus* exhibited a similar but more striking tip localization of CDP II (Fig. 4). This pattern was maintained even when these apices were transformed into appressoria (Fig. 4f), a characteristic response of this phytopathogenic fungus to topographical stimuli (Corrêa and Hoch 1993, Barja et al. 1998). No staining patterns occurred when similar germlings were incubated with preimmune serum as control.

It is widely recognized that fungi grow by apical extension due primarily to fusion of secretory vesicles with the plasma membrane near the cell apex, and that the zone of growth-related activities is located in the apicalmost 100 μm. The growing-tip regions are rich in

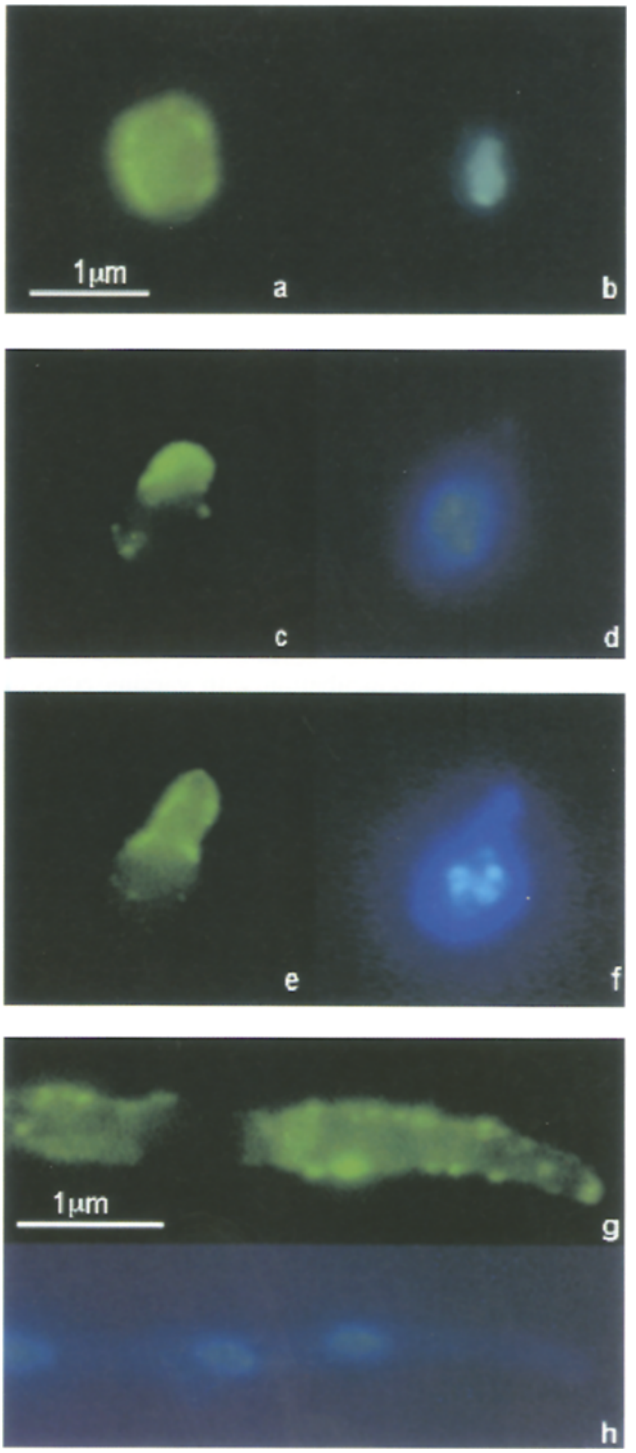

Fig. 3a–h. Distribution of CDP II during conidial germination in *N. crassa* as visualized by immunofluorescence with antibodies prepared against *A. arbuscula* CDP II. **a** and **b** Conidia (0 h); **c** and **d** after 2 h of germination; **e** and **f** after 6 h of germination; **g** and **h** hyphal tip after 12 h of growth. FITC immunofluorescence of CDP II (**a**, **c**, **e**, and **g**) and 4′,6-diamidino-2-phenylindole staining of nucleic DNA (**b**, **d**, **f**, and **h**). Immunoanalogue of CDP II was mostly concentrated in the apical regions of germ tubes (2–6 h) where it appeared as a "uniform cap". In hyphal apices (12 h) staining was characterized more often as "dots"

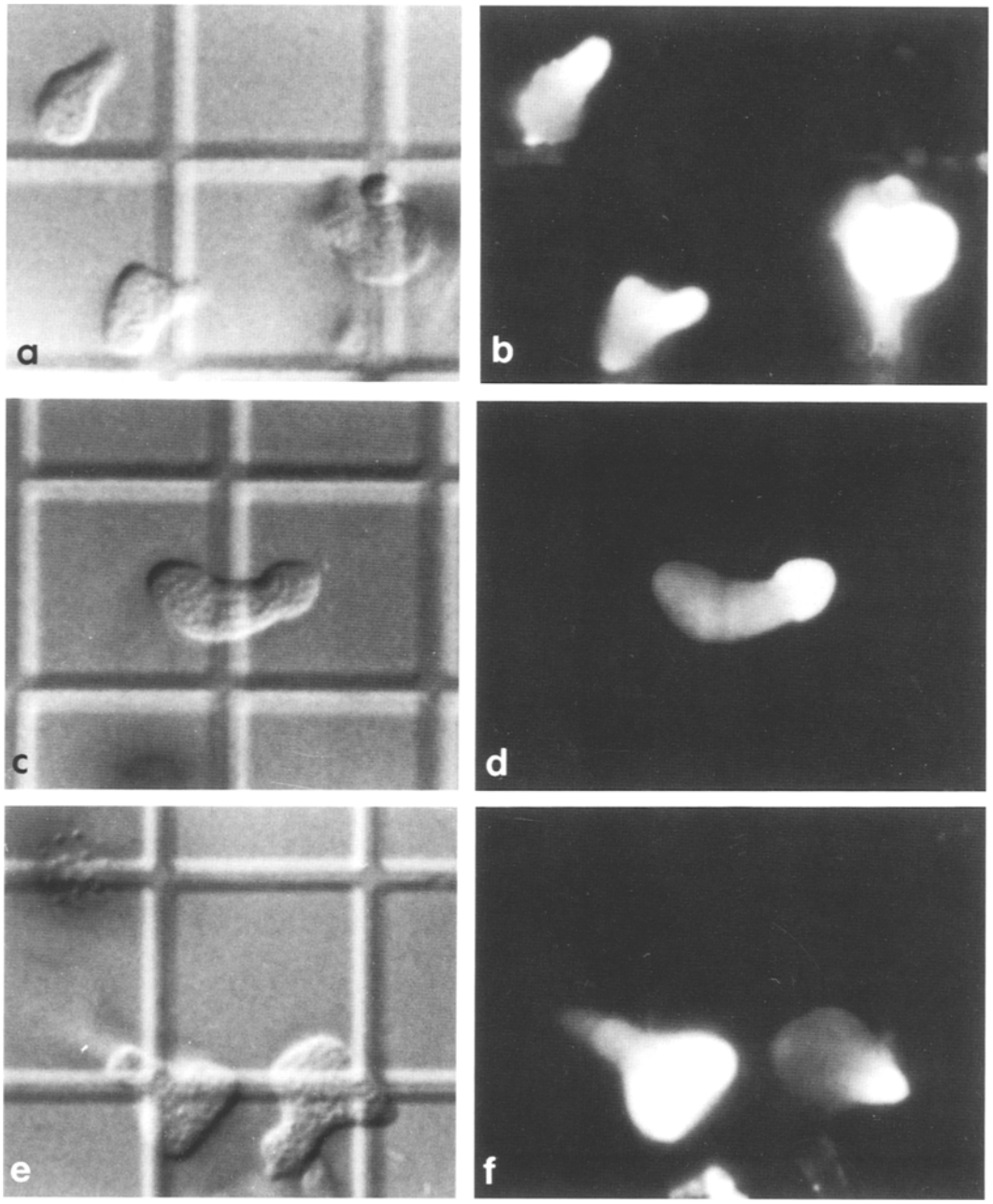

Fig. 4a–f. Distribution of CDP II in formaldehyde-fixed *U. appendiculatus* germ tubes after 3 h of germination visualized with the antibodies specific to *A. arbuscula* CDP II antigen. **a**, **c**, and **e** Nomarski images of germlings showing morphological characteristics. **b**, **d**, and **f** Immunofluorescence of CDP II antigen exclusively concentrated in the tip of germ tube

various cytoskeletal components, most importantly the microfilaments (e.g., actin) and microtubules (Hoch et al. 1987; Barja et al. 1991a, b, 1993; Turian et al. 1992). We previously demonstrated that the tip region in *A. arbuscula* is very rich in CDP II and actin filaments (Huber and Ojha 1994) and suggested a functional relationship between these two proteins. Measurements of Ca^{2+} in growing hyphae have indicated that they maintain a tip gradient of cytoplasmic Ca^{2+} which is essential for growth (Hyde and Heath 1997). The same phenomenon has also been noted when branch initials are formed. Although branch formation does not exhibit elevation of Ca^{2+} in the zone of initiation in the support hyphae but as the branches develop a Ca^{2+} gradient is formed (Hyde and Heath 1997).

The colocalization of cytoskeletal proteins (Hoch et al. 1987; Barja et al. 1991a, b, 1993; Turian et al. 1992), CDP II (Huber and Ojha 1994 and this report), and tip gradient of Ca^{2+} in the dynamic region of fungal apices (Hyde and Heath 1997) reveals the possibilities of interactions between these components and thus involvement of CDP II in the maintenance of the plasticity needed for the apical extension of hyphae. Indeed, the extensively studied calpains, of which CDP II is a functional analogue, show a strong preference for cytoskeletal proteins as substrates (Fox et al. 1985, Billger et al. 1988, Goll et al. 1991). Considering the need for constant remodeling of apical cytoskeletal interactions either between themselves or with the plasma membrane we suggest that the Ca^{2+}-dependent protease plays a crucial role in the process of apical growth. The tip localization of CDP II analogues in *N. crassa* and *U. appendiculatus* points to the remodelling role of CDP II in the tip growth of filamentous fungi (Huber and Ojha 1994, Huber et al. 1994).

Acknowledgments

F.B. dedicates this article to his father, Amador Barja, on the occasion of his 80th birthday. We thank Professor Reto Strasser for his interest in the project and his encouragement, Mrs. A. Cattaneo and M.-L. Chappuis for excellent technical help, Mrs. M. Vautravers for photographic work, and Mrs. A. Fehr for typing the manuscript. We also express our gratitude to the Academic Society of Geneva as well as Novartis-Jubiläums-Stiftung and the Funds Constantin Topali for financial support.

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